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PRINCIPAL INVESTIGATOR: Pappanaicken R. Kumaresan, Ph.D.

CONTRACTING ORGANIZATION: University of California, Davis
Davis, CA 95618

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Pappanaicken R. Kumaresan, Ph.D.

Email: pkumaresan@ucdavis.edu

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14. ABSTRACT

An emerging problem in chronic phase CML is molecular persistence. It is mainly due to the quiescent stem cell population that are completely insensitive to Imitinib therapy. We have developed a novel immunotherapy against CML. We have screened One-Bead-One-Compound (OBOC) combinatorial libraries and identified cyclic peptide ligands that are bind CML cancer cells. These ligands will then be ligated to the N-termini of the engineered Fc fragment of human immunoglobulin in a site-specific manner. We hypothesize that these cancer targeting ligand-Fc fragment conjugate (we call it "ligand-body") will bind to CML cells via the peptide or peptidomimetic ligand domain and the Fc immunoglobulin domain will be used to harness the anti-cancer innate immunity against CML cells in vivo. The innate immune system includes immune effector cells such as NK cells, NKT cells, macrophages and leukocytes and complement system. As mentioned in Aim1 and Aim 2, we have modified Fc domain for specific N-terminal ligation and produced modified protein for Ligand-body production. In the second year, functional characterization for the ligand-body will be performed.

15. SUBJECT TERMS
None listed.

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Novel targeted immunotherapy for CML blast cells
(Fist year Progress report (June-2007 to May 2008)

1. INTRODUCTION: An emerging problem in chronic phase CML is molecular persistence. It is mainly due to the quiescent stem cell population that are completely insensitive to Imitinib therapy. We have developed a novel immunotherapy against CML. We have screened One-Bead-One-Compound (OBOC) combinatorial libraries and identified cyclic peptide ligands that are bind CML cancer cells. These ligands will then be ligated to the N-termini of the engineered Fc fragment of human immunoglobulin in a site-specific manner. We **hypothesize** that these cancer targeting ligand-Fc fragment conjugate (we call it "ligand-body/ chembody") will bind to CML cells via the peptide or peptidomimetic ligand domain and the Fc immunoglobulin domain will be used to harness the anti-cancer innate immunity against CML cells in vivo. The innate immune system includes immune effector cells such as NK cells, NKT cells, macrophages and leukocytes and complement system.
The **specific Aims** of the project are:
Aim1: Construction of Fc with N-terminal cysteine for site-specific ligand ligation. Using site-directed mutagenesis, cysteine will be incorporated into the N-terminal end of Fc fragment. DNA construct containing CH2 and CH3 regions of the human IgG1 (corresponding to Fc fragment) from genomic DNA amplified by PCR and cloned with CD5 leader sequences called pCD51neg1 vector has been provided to us as a kind gift from Dr. Brain Seed. He and his associates have used it to construct several chimeric-human IgG fusion protein (22) (reprints attached). For large-scale production of modified human IgG Fc fragment will be performed, by transfecting CsCl2 purified CD5Lneg1 pDNA into COS-7 cells and collect the serum free supernatant on day 4, day 6 and day 8. Modified human IgG1-Fc fragment will be purified by protein-A-agarose affinity column. Glyoxylyl-linker-ligand (identified from aim 2) will be conjugated to the N-terminal cysteine via a site-specific thiazoline ring formation reaction to form ligand-body (aim 3).
Aim2: Identification and optimization of CML specific ligands.
Aim3: Preparation and biological characterization of ligand-body.

2. BODY: Chemical antibodies (also noted as chembody) were made by conjugating targeting ligands to immunoglobulin (IgG). Site specific and non-site specific conjugation methods were used to make chembodies. Progress report is broadly categorized in to two sub headings:

(1) Construction and purification of N-terminal cysteine mutated Fc-fragment of human IgG1 (hIgG1-Cys-Fc).
(2) Conjugation of targeting ligands to hIgG1-Cys-Fc, hIgG1-Fc and hIgG1
    2.1: N-terminal cysteine site specific conjugation
    2.2: non-site specific conjugation.

(1) Construction and purification of N-terminal cysteine mutated Fc-fragment of human IgG1 (cys-Fc-hIgG1).
Conjugation of the targeting ligand at N-terminal site of the IgG is preferred to protect IgG biological properties. Amine group conjugation at amino terminal is often interfered by the primary amine groups of lysine. To make it site specific a cysteine amino acid was introduced at the N-terminal by site directed mutagenesis (promega). N-terminal cysteine was used for site-specific conjugation.

Site directed mutagenesis: Human IgG1-Fc fragment expressing pFuse vector (Fig.1) (Invivogen) has been used to make N-terminal cysteine (Ig). The primers are designed and site directed mutagenesis was performed as described by protocol (Quick change ^{TM} site-directed mutagenesis kit, cat# 200518, Promega). The primer sequences are shown in Table-1. Both primers bind at the same position of pFuse vector (611-653) and first amino acid after the signal sequence is mutated to cysteine. The native pDNA served as templates for PCR was digested by DpnI restricted endonuclease (RE). The RE digested PCR product was transformed to high efficiency competent cells (bluescript) and bacterial clones were selected on LB medium containing Zeocin 200 μg/mL. Plasmid DNA was isolated from positive clones and sequenced at UC Davis sequence facility to

conform the mutation. Five clones were selected and labeled as hIgG1-cys-21 to 25 respectively. One of the sequence alignment are shown in Table-1. Plasmid DNA of the mutated clone was used to transfect mammalian cell lines CHO (Chinese Hamster Ovary) by lipofectamine-2000 and stably hIgG-Fc expressing clones were selected by growing in DMEM medium supplemented with 10% FBS and zeomycin antibiotic at 500 µg/mL.

1.1 Mutated human IgG1-Fc (hIgG1-Cys-Fc) **protein production:** Expression level of hIgG1-Cys-Fc fragment was determined by performing sandwich ELISA method. Serum free supernatant of positive clones

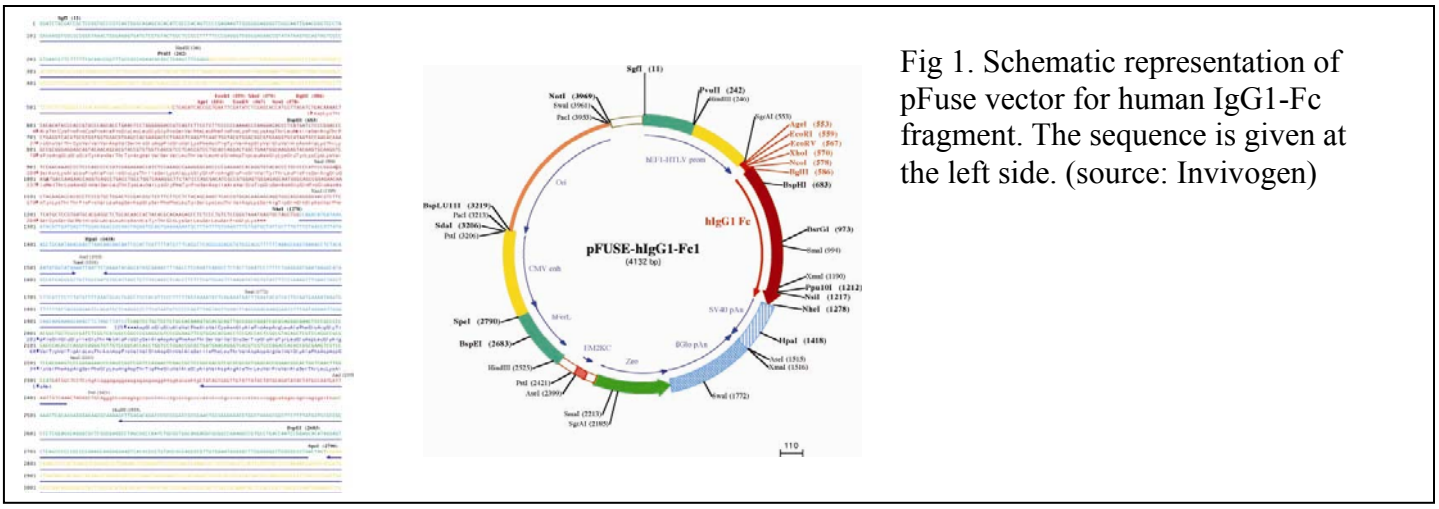

Fig 1. Schematic representation of pFuse vector for human IgG1-Fc fragment. The sequence is given at the left side. (source: Invivogen)

were collected and incubated for 1 h in protein-G coated plates (Pierce biotechnology). Supernatants were aspirated and alkaline phosphatase (AP) conjugated anti-human Fc specific antibody (Bethyl labs, TX) was added and incubated for 30 min at room temperature. Three washes were given with PBS, and DAB substrate was added to develop color. Based on the color intensity, highly expressing positive clones were selected for hIgG-Cys-Fc protein production. Serum free supernatants from highly expressing clones were collected and hIgG1-Cys-Fc protein was purified by Protein-G affinity columns (Biorad). The purity of the protein was checked by separating in 10% SDS-PAGE reduced electrophoresis and transferred to a nitrocellulose membrane. Another gel was stained with Coomassie blue and gel pattern was documented.

Table 1. Mutagenesis primer sequences

Primer	Sequences 611-653 of pFUSE-hIgG1-FC2 (Invivogen)
Fc-cys-FP (Forward primer)	5'-CTT GTC ACG AAT TCG TGC TCG GCC ATG GTT AGA TCT GTG GAG-3'
Fc-cys-Rp (Reverse primer)	5'-CTC CAC AGA TCT AAC CAT GGC CGA GCA CGA ATT CGT GAC AAG-3'
Sequence Alignment	605 615 625 pFUSE-hIgG TTGCACTAAG TCTTGCACTT GTCACGAATT hIgG1-cys-25 TTGCACTAAG TCTTGCACTT GTCACGAATT 635 645 655 CGATATCGGC CATGGTTAGA TCTGACAAAA CGTGCTCGGC CATGGTTAGA TCTG------
Sequences of pFuse-hIgG1-Fc2 and hIgG1-Cys-Fc-25 and its alignments are in the table-1. The sequence alignments are done by using Bioedit software. Mutated "Cys" is shown in red color.	

Western blot was developed by AP-conjugated goat anti-human Fc-specifc antibody (Fig 2) using chemiluminescence kit (perkin-elmer, CDP star kit). In the reducing gel, ~25 kD band showed positive to anti-human IgG-Fc specific antibodies suggests that the expressed protein is hIgG-Cys-Fc fragment. Because of the reducing gel it ran in monomeric form at ~25 kD. The estimated MW of the protein is hIgG-Cys-Fc is ~ 50 kD. After conforming with western blot analysis, hIgG1-cys-Fc protein has taken for conjugation studies.

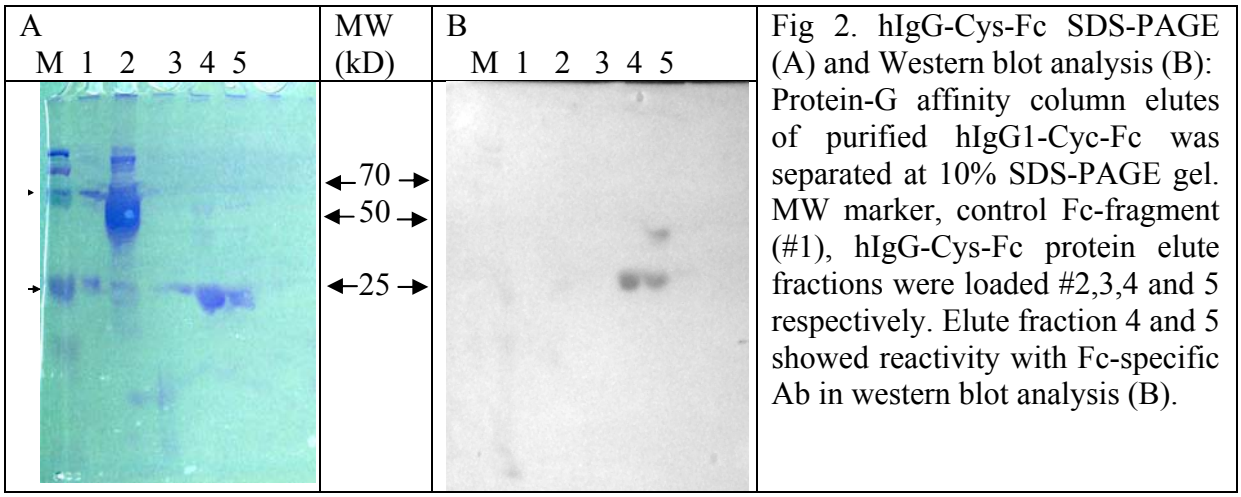

Fig 2. hIgG-Cys-Fc SDS-PAGE (A) and Western blot analysis (B): Protein-G affinity column elutes of purified hIgG1-Cyc-Fc was separated at 10% SDS-PAGE gel. MW marker, control Fc-fragment (#1), hIgG-Cys-Fc protein elute fractions were loaded #2,3,4 and 5 respectively. Elute fraction 4 and 5 showed reactivity with Fc-specific Ab in western blot analysis (B).

(2) Conjugation of targeting ligands to hIgG1-Cys-Fc, hIgG1-Fc and hIgG1

2.1 Synthesis of targeting ligands

2.2 N-terminal cysteine site specific conjugation

2.3 non-site specific conjugation

The LLP2A-a lymphoma targeting ligand was selected to make a protype of chembody. LLP2A is a targeting ligand for α4β1 integrin receptor which is over expressed in lymphomas. The exact role of α4β1 integrin in cancer remains to be completely elucidated. It is hypothesized that it plays a major role in facilitating metastatic disease. For instance, it has been shown to be expressed only on proliferating cells during tumor development, not on quiescent cells. In B-CLL, α4β1 integrin is linked to drug resistance and apoptosis resistance. In a murine model of myeloma, a monoclonal antibody to α4β1 integrin was shown to inhibit tumor growth and metastasis, without any effect on normal hematopoietic cells. Based on these findings, α4β1 is a promising target for the treatment of both cancer and certain inflammatory diseases.

2.1 Synthesis of LLP2A targeting ligand: LLP2A-maleimide and LLP2A-glyoxylyl was synthesized using synthetic FMOC protected chemistry. After synthesis the LLP2A-derivatives were purified through C18, HPLC column. MALDI-TOF mass spectrometry revealed its mass is 1550 Da which is close to the calculated mass. The structure and schematic representation of LLP2A-maleimide synthesis is shown in fig 3.

2.2. N-terminal cysteine site specific conjugation with LLP2A-maleimide :

Maleimide can react with sulfhydryl functional groups and form a stable thio ether bonds. Maleimide reactions are specific for sulfhydryl groups in the pH range 6.5-7.5, at pH 7.0, the reaction of the Maleimide with sulfhydryls proceeds at a rate 1000 times greater than its reaction with amines. This type of conjugation is beneficial to proteins which has few cysteines especially immunoglobulins (Igs). The cysteines present in Ig proteins are either present in the inner grove or formed S-S double bonds with other cysteines. By using reducing agents such as dithiothreital (DTT), β-mercaptoethanol, TCEP up to eight functional –SH groups can be produced for conjugation. The disulfide bond that holds two heavy chains together has been in use to couple AP or HRPO enzymes to conjugate in antibody for immunohistochemistry. Conjugation done using hinge-area –SH groups will orient the attached protein or other molecules away from the antigen binding regions, thus preventing blockage of these sites and preserving activity. At the same time conjugation at hinge region and CH2 domain of the Abs will diminish other functions of Ab such as recruitment of host immune cells to fight against abnormal cells. This can be overcome by introducing cysteine amino acid at the N-terminal end with free –SH functional group which is an ideal for site specific conjugation. We have used cysteine introduced hIgG-Fc (hIgG1-cys-Fc) for conjugating targeting ligands either by maleimide conjugation or by glyoxylyl conjugation.

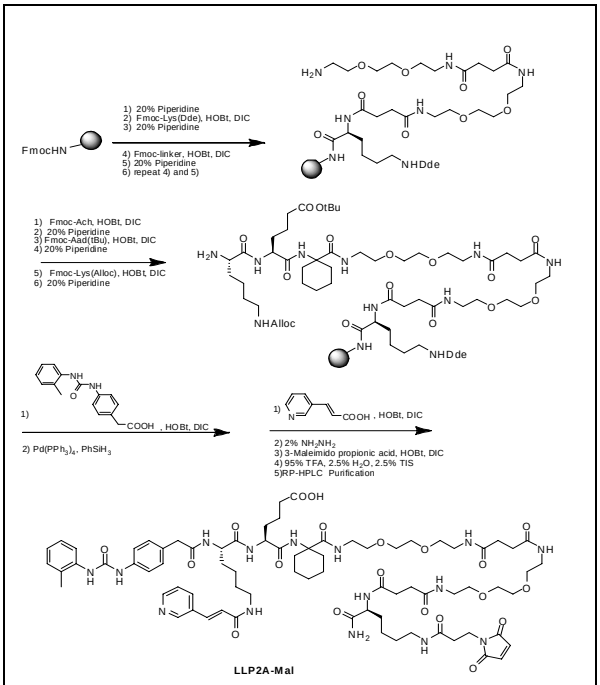

Fig. 3: LLP2A-maleimide synthesis

2.3. Maleimide conjugation:

200 µg of hIgG1-Cys-Fc (4 n moles) with 40 nmoles of LLP2A-maleimide in 100 µL volume of 0.1 M $NaHPO_4$, 150 mM $NaCl_2$, 10 mM EDTA, pH 7.2 and incubated for 4 h at room temperature. After conjugation free LLP2A was removed by Sephadex G-50 spin columns. The schematic representation of site-specific conjugation LLP2A-maleimide is shown in Fig 4. The purified product was separated in 10% SDS-PAGE electrophoresis and western blot (Fig. 5) was performed as described in section 1.2. In the SDS-PAGE gel, the LLP2A-conjugated hIgG1-Cys-Fc showed higher mass than hIgG1-Cys-Fc suggests that the increased mass is due to conjugation LLP2A (Fig 5A).

1.2 Mutated human IgG1-Fc (hIgG1-Cys-Fc) **protein production:** Expression level of hIgG1-Cys-Fc fragment was determine by performing sandwich ELISA method. Serum free supernatant of positive clones were collected and incubated for 1 h in protein-G coated plates (Pierce biotechnology). Supernatants were aspirated and Alkaline phosphatase (AP) conjugated anti-human Fc specific antibody (Bethyl labs, TX) was added and incubated for 30 min at room temperature. Three washes were given with PBS, and DAB substrate was added to develop color. After verifying the LLP2A conjugation LLP2A-hIgG1-Cys-Fc has taken for further chembody functional studies.

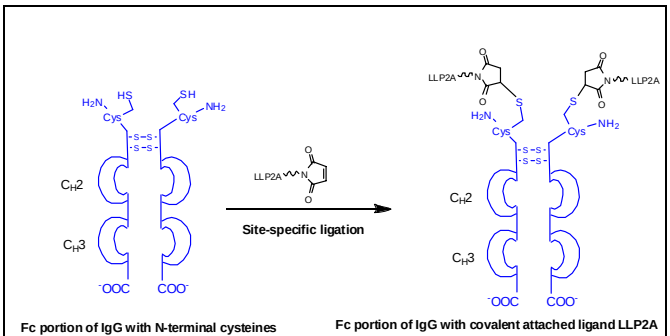

Fig 4. Schematic representation of LLP2A-chembody

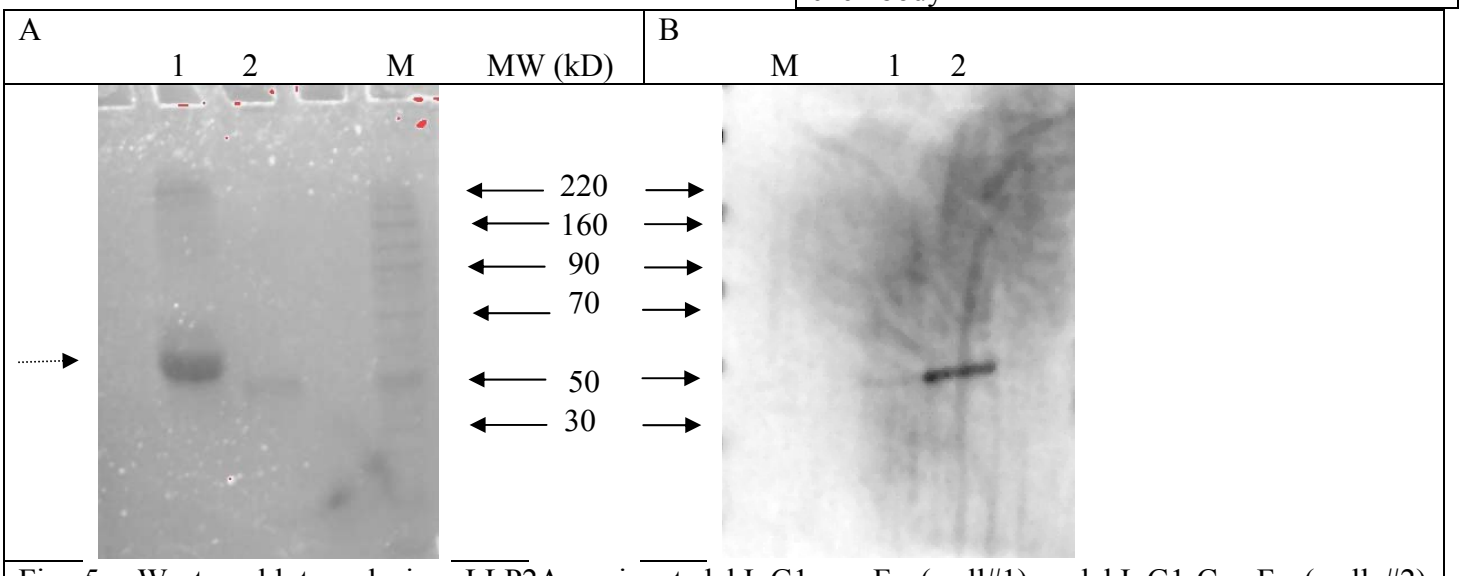

Fig. 5. Western blot analysis: LLP2A-conjugated hIgG1-cys-Fc (well#1) and hIgG1-Cys-Fc (well #2) separated in 10% non-reduced SDS-PAGE electrophoresis (A) and transferred to nitrocellulose membrane for western blot analysis (B). LLP2A-hIgG1-Cys-Fc band showed higher mass compared to hIgG1-Cys-FC due to conjugation LLP2A targeting ligands (⋯►)(A). In the western, Fc-specific goat anti-human antibodies binds strongly to hIgG1-Cys-Fc and weaker binding to LLP2A-hIgG1-Cys-Fc suggests that LLP2A conjugation blocks goat anti-Fc Ab binding domain of the human Fc.

3. KEY RESEARCH ACCOMBLISHMENTS:

1. Modified Fc domain of the human IgG (hIgG-cys-Fc) was made by site-directed mutagenesis.
2. hIgG-cys-Fc was stably transfected and purified from the cell culture supernatant.
3. A prototype of ligand-body (chembody) was made by conjugating with lymphoma targeting ligand (LLP2A).
4. The conjugation was verified by SDS-PAGE and western blot analysis.

4. REPORTABLE OUTCOMES:

Pappanaicken R. Kumaresan, Aimin Song, Jan Marik, Juntao Luo, Kit S. Lam (2008) Evaluation of ketone-oxime conjugation method for developing On-demand cleavable immunoconjugates for radio immunotherapy. Bioconjugate chemistry, (Inpress).

5. CONCLUSION: We have successfully constructed chemical antibodies against lymphoma. Functional studies of the chemical antibodies such as tumor targeting and effector cell recruitment will be evaluated. The same studies will be repeated by conjugating with CML-blast targeting ligand against minimal residual disease CML cells.

6. REFERENCE: